Title

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Journal

Journal of the Institute of Brewing, 123(3)

ISSN

0046-9750

Authors

Kanyer, AJ
Bornhorst, GM
Marco, ML
et al.

Publication Date

2017-07-01

DOI

10.1002/jib.439

Peer reviewed

Is beer a source of prebiotics?

Abigail J. Kanyer, Gail M. Bornhorst, Maria L. Marco and Charles W. Bamforth*

Department of Food Science and Technology, University of California, Davis, CA 95616, United States of America

*Correspondence to: Charles W. Bamforth, Department of Food Science & Technology, University of California, Davis, CA 95616-8598, USA.

E-mail: cwbamforth@ucdavis.edu

Abstract

Beer contains low molecular weight β-linked oligosaccharides that originate from the degradation of β-glucan in the barley cell wall during malting and mashing. Over 90% of these oligosaccharides contain three or four glucosyl units. They remain intact through a static oral, gastric and small intestinal *in vitro* human digestive system model, indicating that they should be available to beneficial organisms known to be present in the human large intestine. Several intestine-associated *Lactobacillus* strains were shown to be capable of growth on these β-linked oligosaccharides, thereby leading us to tentatively propose that these compounds may represent prebiotics.

Key words: in vitro digestion, β-glucan, gut microbiota, Lactobacillus, oligosaccharides, prebiotics

Introduction

The wall surrounding the cells in the starchy endosperm of barley comprises 75% β-(1,3)(1,4)-glucan, 20% arabinoxylans, with the balance comprising protein, acetic acid and ferulic acid (*6, 9, 11*). Most of the focus on the walls has always been on ensuring their removal and digestion to prevent processing challenges in brewing by minimizing the levels of viscous glucan and pentosan surviving into beer, such materials leading to problems with filtration and stability (*2, 20*). However it has been recognized that these molecules may represent soluble fiber in beer (*3, 5, 8, 15, 22*).

The enzymic digestion of the cell walls has been extensively researched (*4*). In relation to the degradation of β-glucan, it is recognized that the key enzyme catalyzing the hydrolysis of the molecule is endo- β-(1,3)(1,4)-glucanase, which specifically targets β1-4 bonds adjacent to β1-3 bonds on the reducing side (*31*). As barley β-glucans largely comprise regions with a β1-3 link every third or fourth bond, the remainder being β1-4 linkages, 91-97% of glucan hydrolysis products by this enzyme are oligosaccharides containing three or four glucosyl residues.

Although there are a series of exo-glucanases and a β-glucosidase capable of continuing the degradation, theoretically making glucose the final hydrolysis product, it appears that these enzymeshave only very limited action during malting and mashing, likely on account of a poor affinity for the tri- and tetrasaccharides (*18, 21*). These β-glucan oligosaccharides (BGOs) are not fermentable by brewing yeast and accordingly they can be present in substantial quantities in beer (*22*). A variety of beers examined via HPLC displayed significant levels of high molecular

weight pentosans (0.88-1.79 mg/mL), and a mixture of high and low molecular weight β-glucans (4.0-6.0 mg/mL) *(22)*.

It has been suggested that the BGOs might be prebiotics (*5*) viz. a "non-digestible food ingredient that beneficially affects the host by selectively stimulating growth and/or activity of one or a limited number of bacteria in the colon" (*12*). However this assignment has remained as "putative" as hitherto β-glucan oligosaccharides have not been shown to survive the digestive process through to the large intestine, and there has been limited data on their ability to serve as substrates for the beneficial microorganisms of the gut. The present investigation addresses these issues.

Materials and Methods

Production of β-glucan oligosaccharides

Oligosaccharides were produced by the hydrolysis of barley β-glucan using endo-barley-β-glucanase. Low viscosity, high purity β-glucan flour (Megazyme Cat No.: P-BGBL; Lot # 100401) was hydrolyzed using endo- β-(1,3)(1,4)-glucanase (Megazyme Cat. No: E-LICHN; Lot #70502a) employing the procedure of Sims et al (*29*). β-Glucan (1.0g) was dissolved in 50mL of deionized water by heating at 90˚C for 15min with stirring. The sample was cooled to room temperature and endo-barley-β-glucanase (25μL; 1000units/ml) was added. Hydrolysis was allowed to proceed for 5h at 50˚C and it was halted by boiling for 10min followed by centrifugation (3200g, 4˚C, 20min).

Removal of glucose

The removal of glucose was achieved by the addition of brewing yeast. A 10%w/v slurry of yeast (Lalvin EC-1118; Canada; Cat. No.: YST-LALV-EC1118-5G; *Saccharomyces cerevisiae*) was added to the β-glucan hydrolysate and allowed to ferment at room temperature (20˚C) for 3-5 days. The sample was then cooled to 4˚C for 48h before centrifugation (1800 x g, 4˚C, 15min). The supernatant was collected and sterile filtered (0.2micron syringe) before use.

Thin layer chromatography (TLC)

Comparative TLC was used to ascertain the extent of enzymatic degradation of β-glucan into oligosaccharides (*22*). Reference standards were D(+)-cellobiose (Acros Organics, Geel, Belgium; Cat No.: 108460050), D(+)-cellotriose (Megazyme; Cat No.: O-CTR50), D(+)-cellotetraose (Megazyme; Cat No.: 0-CTE50). Plates were spotted (2.5µL of hydrolysates and standards, the latter at 5.0mg/mL) onto a glass backed silica TLC plate (Merck; Darmstadt, Germany; TLC Silica gel 60 plate; Cat No: 1.05721.0001) and run in a solution comprising ethyl acetate (60mL), methanol (20mL), glacial acetic acid (15mL) and water (5mL). An anisaldehyde stain (26mL ethanol, 1.5mL *p*-anisaldehyde [Acros Organics; Cat No.: 104801000], 1.5mL sulfuric acid and 0.5mL glacial acetic acid) was sprayed directly on to the plates immediately after running. The plates were heated at 100˚C in an oven for 10min. After 10min, the plates were removed, sprayed a second time with the stain, and returned to the oven for an additional 20min.

In Vitro Digestion Model

The BGO solutions were digested using a static *in vitro* digestion model including oral, gastric, and small intestinal phases (*7, 27*). The three simulated digestive solutions – saliva, gastric fluid and intestinal fluid -- were prepared prior to digestion with the omission of the enzymatic reagents, which were added to the formulations immediately prior to the use of each solution.

The simulated saliva comprised mucin from porcine stomach (Sigma; St. Louis, MO; Cat No.: M2378-500G), α-amylase (MP Biomedicals, LLC; Burlingame, CA; Cat No.: 100447), NaCl, KCl and $NaHCO_3$ (Fisher; Waltham, MA; Cat No: S671, P217, S223) dissolved in deionized

water (pH 7). The simulated gastric fluid comprised mucin from porcine stomach (Sigma; Cat No.: M2378-500G), pepsin (Fisher; Cat No.; P53-100) and NaCl dissolved in deionized water (pH 1.8). The simulated intestinal digestion fluid comprised pancreatin from porcine pancreas (Sigma; St. Louis, MO; Cat No.: P3292), bile extract porcine (Sigma; Cat No.: B8631-100G) and $NaHCO_3$ dissolved in deionized water (pH 6.5). The quantities of the individual reagents used in each formulation is outlined in Table 1. The pH of all solutions was adjusted prior to addition of enzymes.

Simulated saliva (3.33mL) was mixed with 5mL of oligosaccharide solution prepared as above and hand-shaken for 30s. Following this simulated oral digestion, 6.66mL of simulated gastric fluid was added to the sample. The sample was incubated in a heated water bath for 40min (110 rpm, 37˚C). The pH of the sample was then monitored to ensure it was within the range of pH 1.8 – 2. If the pH of solution was out of range, it was adjusted by dropwise addition of a 1M HCl solution. The solution remained in the shaking water bath for an additional 80min, for a total of 120min of simulated gastric digestion. A 1.0mL sample was collected and neutralized to pH 7 with 0.1M NaOH solution and stored at -18˚C until analysis. Directly following the 120min of gastric digestion, 10mL of simulated intestinal fluid was added to the sample. The pH of the sample was adjusted to 6.5, if necessary. The sample was incubated for an additional 120min (110 rpm, 37˚C). After this period, a 1.0mL sample was collected and neutralized to pH 7 with 0.1M NaOH solution and stored at -18˚C until analysis by TLC. Digestions were completed in triplicate.

Growth of bacteria on oligosaccharides

Seven strains of *Lactobacillus* species (Table 2) were tested for their ability to grow on BGOs. *Lactobacillus* were cultured overnight in MRS medium (MRS; 0.5g Protease Peptone, 0.05g Tween 80, 1g carbon source, 0.25g yeast extract, 0.1g K_2HPO_4, 0.415g sodium acetate trihydrate, 0.1075g ammonium citrate tribasic, 0.005g $MgSO_4$, 0.0025g $MnSO_4$ in 46mL of deionized water) with glucose as the carbon source at 30˚C, collected via centrifugation (7500 x g; 20˚C; 5min), washed twice with sterile phosphate-buffered saline (PBS) solution and re-suspended in PBS. MRS medium without glucose, MRS(NC), was used as basal medium lacking any additional carbon sources. This MRS(NC) medium was used as a negative control. Sterile glucose was added to the basal medium to create a 2% w/v glucose medium, MRS(+), to be used as a positive control. The yeast-treated β-glucan hydrolysate (ds) was sterilized by passage through a 0.2µm filter and added to the basal medium (MRS(NC)) to create a 2%w/v BGO medium, MRS(BGO), with the β-glucan oligosaccharides as the only carbon source. The washed and suspended cultures were added to each medium to an optical density (OD) at 600nm of 0.0.05 and incubated at 30˚C for 48h. Proliferation of *Lactobacillus* on each substrate was determined by assessing the $OD_{600\ nm}$ of the cultures in a microplate via a spectrophotometer microplate reader (Biotek; Winooski, VT, USA). Cultures were run in triplicate.

Results and Discussion

Hydrolysis of β-glucan

Hydrolysis of barley β-glucan catalyzed by *endo*–1,3 (4)- β-glucanase yielded a mixture of low molecular weight oligosaccharides (Fig 1), as previously observed (*16, 29, 31*). Comparison with commercially available standards of D(+)-cellotriose (DP 3) and D(+)-cellotetraose (DP 4) indicates that the most abundant BGO product is a trisaccharide.

Treatment of the BGO with *S. cerevisiae* to remove glucose did not result in the loss of the DP 3-4 oligosaccharides (data not shown). This result confirms that *S. cerevisiae* is incapable of utilizing the DP 3-4 oligosaccharides, meaning that these compounds will survive into beer.

Survival of BGOs in a model digestion system

A static *in vitro* digestion model was used to determine the survival of the BGO compounds through the process of digestion (Fig 2). The DP 3-4 BGOs survived both the enzymatic stress and low pH conditions associated with the digestive process, suggesting they are available for consumption by microbiota associated with digestion and the lower gastrointestinal (GI) tract. This satisfies the first requirement for a compound to be considered as a true prebiotic (*13*).

Growth of *Lactobacillus* spp. on β-glucan oligosaccharides

The prebiotic potential of the BGO compounds was tested by performing growth studies using seven strains of *Lactobacillus*: *Lactobacillus reuteri* ATCC 23272, *L. casei* BL23, *L. fermentum* B6, *L. plantarum* NCIMB8826, *L. rhamnosus* GG, *L. plantarum* B1.3 and *L. plantarum* B1.1. These organisms were selected based on their origins with respect to fermented food and beverages and the human digestive tract (Table 2). All cultures exhibited robust growth on glucose (data not shown). Five of the seven strains tested, *L. casei* BL23, *L. fermentum* B6, *L. plantarum* NCIMB8826, *L. rhamnosus* GG, and *L. plantarum* B1.1, were capable of utilizing and growing on the BGOs in the absence of any other carbon source (Fig 3). The data from the present study is representative of multiple growth experiments with BGOs.

L. rhamnosus is one of the most abundant species of common *Lactobacillus* species found in the human gastrointestinal tract and one of the most commonly studied organisms in probiotic research (*1, 32*). *L. rhamnosus* GG and other *L. rhamnosus* strains were previously shown to utilize β-glucan-derived oligosaccharides from barley as a substrate for growth (*29*). Jaskari et al (*19*) showed that comparable oligosaccharides derived from oat β-glucan have the same impact. In the present study, four other strains grew on BGOs. However, the capacity to metabolize BGO was not common to all lactobacilli and even different strains of the same species as indicated by the lack of growth of *L. plantarum* B1.3 in contrast to the robust growth of *L. plantarum B1.1* and *L. plantarum* NCIMB8826. Russo et al (28) identified *bgl*, lp_3629 as a key gene in *L. plantarum* WCFS1 that codes for a β-glycosidase, thus enabling the metabolism of β-glucan hydrolysates. Additional works should aim to elucidate other essential genes responsible for the utilization of β-glucan and BGOs by various lactobacilli.

Snart et al (*30*) speculated on the potential of β-glucan hydrolysis products to be substrates for the growth of beneficial lactobacilli in the gut, although this idea was challenged (*17*). Both studies indicated that high molecular weight β-glucans, as opposed to BGOs, are more likely to reach the lower intestine. However, as observed earlier, the latter is usually of little significance with respect to beer as brewers strive to minimize the levels of the more viscous β-glucan fractions in beer. Neither of these studies investigated the potential for arabinoxylans to serve as a prebiotic. Neither did they incorporate a digestion tract model as part of their investigations.

The present study shows that oligosaccharide degradation products resulting from the hydrolysis of β-glucan will survive into the large intestine and will support the growth of beneficial organisms. As a rule of thumb it can be calculated that a human adult will take in 2 liters of water from food and drink daily, with salivary, gastric, biliary, pancreatic and intestinal fluids contributing an additional 8 liters (*10*). Approximately 1 liter of the total reaches the colon. Assuming daily consumption of 0.33 liters of beer containing 4mg/mL β-linked oligosaccharide (*22*) as part of the 2 liter daily intake, then the intake to the digestive tract of such oligosaccharides would be at a concentration of 0.67g/L. Assuming no loss of these molecules in the digestive tract and factoring in the two-fold concentration (volume of saliva in comparison to volume entering the colon) then the concentration of BGOs will be 1.34g/L in the colon. This is approximately 20-fold lower than the concentrations used in the growth experiments in this study. Future studies should focus on examining the relationship between oligosaccharide concentration and growth of beneficial bacteria as well as investigating the impact that arabinoxylans may play as additional prebiotics. Previous work on arabinoxylan-degradation products would certainly point to these materials also functioning as prebiotics (*14, 26*).

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Table 1. *Digestive solution recipes (7)*

Materials	Formulations
Initial Sample Volume (Hydrolyzed β-glucan)	5.0 mL
Saliva Formulation	Mucin (1g/L) α -amylase (1.18g/L) NaCl (0.117g/L) KCl (0.149g/L) $NaHCO_3$ (0.21g/L) Adjusted to pH 7 using 1M HCl and 0.1M NaOH 3.33 ml saliva solution : 5.0 mL sample (1:1.5)
Gastric Juice Formulation	Mucin (1.5g/L) NaCl (7.8g/L) Pepsin (1.0g/L) Adjusted to pH 1.8 using 1M HCl 6.66 mL gastric solution : 8.33 mL digestive mixture (1:1.25)
Intestinal Juice Formulation	Pancreatin (2.4g/L) Bile Extract (10g/L) $NaHCO_3$ (16.8g/L) Adjusted to pH 6.5 using 1M HCl and 0.1M NaOH 10 mL intestinal : 14.99 mL digestive mixture (1:1.5)

Table 2. *Lactobacillus spp. used in growth studies*

Organism[a]	Origin	Abbreviation Code
Lactobacillus casei BL23	[b]Unknown[24]	LC
Lactobacillus fermentum B6[c]	Boza (fermented African beverage)	LF
Lactobacillus plantarum B1.1	Ethiopian injera dough (uncooked)	LB1.1
Lactobacillus plantarum B1.3	Ethiopian injera dough (uncooked)	LB1.3
Lactobacillus plantarum NCIMB8826[d]	Human saliva[16]	LPN
Lactobacillus reuteri ATCC 23272[e]	Human intestinal tract[25]	LR
Lactobacillus rhamnosus GG	Human intestinal tract[23]	LGG

a. Unless otherwise indicated, organisms are from the laboratory of Dr. Maria Marco

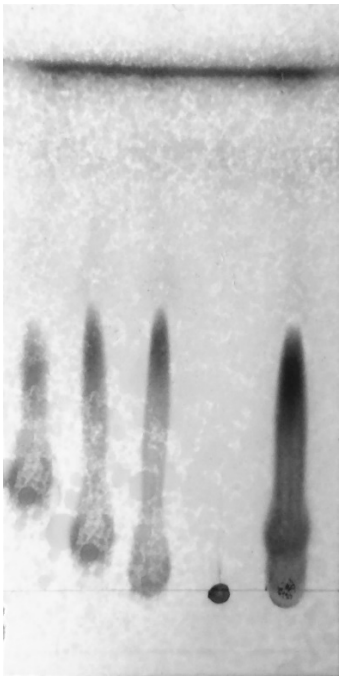
[b] Presumed to be isolated from the human intestinal tract

[c] Received from Dr. Angel Angelov and Dr. Velitchka Gotcheva, University of Food Technologies, Bulgaria

[d] National Collection of Industrial and Marine Bacteria, Aberdeen, UK

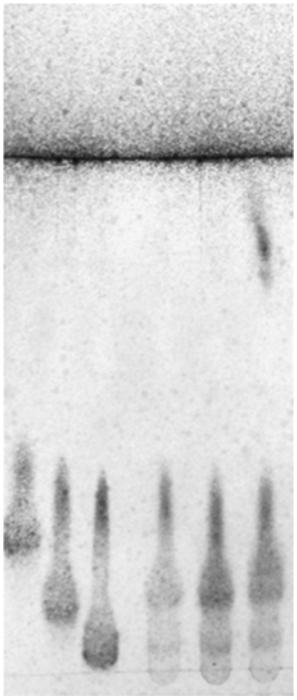
[e] American Type Culture Collection

Figure 1. TLC of unhydrolyzed and hydrolyzed β-glucan. A 2% w/v solution of unhydrolyzed β-glucan (D) was enzymatically degraded using endo–1,3 (4)- β-Glucanase to yield a solution with newly generated, lower molecular weight β-glucan oligosaccharides (E). The hydrolysis products (E) were compared to DP 2 (A), DP 3 (B), and DP 4 (C) standard references (2.5µl, 5mg/ml).



A B C D E

Figure 2. *Thin layer chromatography plate of hydrolyzed and digested β-glucan oligosaccharides***.** Experimental samples (4 – 0.4% w/v hydrolyzed β-glucan; 5 – post-gastric (G) digestion of 2% w/v hydrolyzed β-glucan, final BGO concentration 0.67% w/v; 6 – post-intestinal (I) digestion of 2% w/v hydrolyzed β-glucan, final BGO concentration 0.4% w/v) were compared to standards (1- cellobiose (DP 2) standard, 5 mg/mL; 2 - cellotriose (DP 3) standard, 5 mg/ml; 3 - cellotetraose (DP 4) standard, 5 mg/mL) via TLC.



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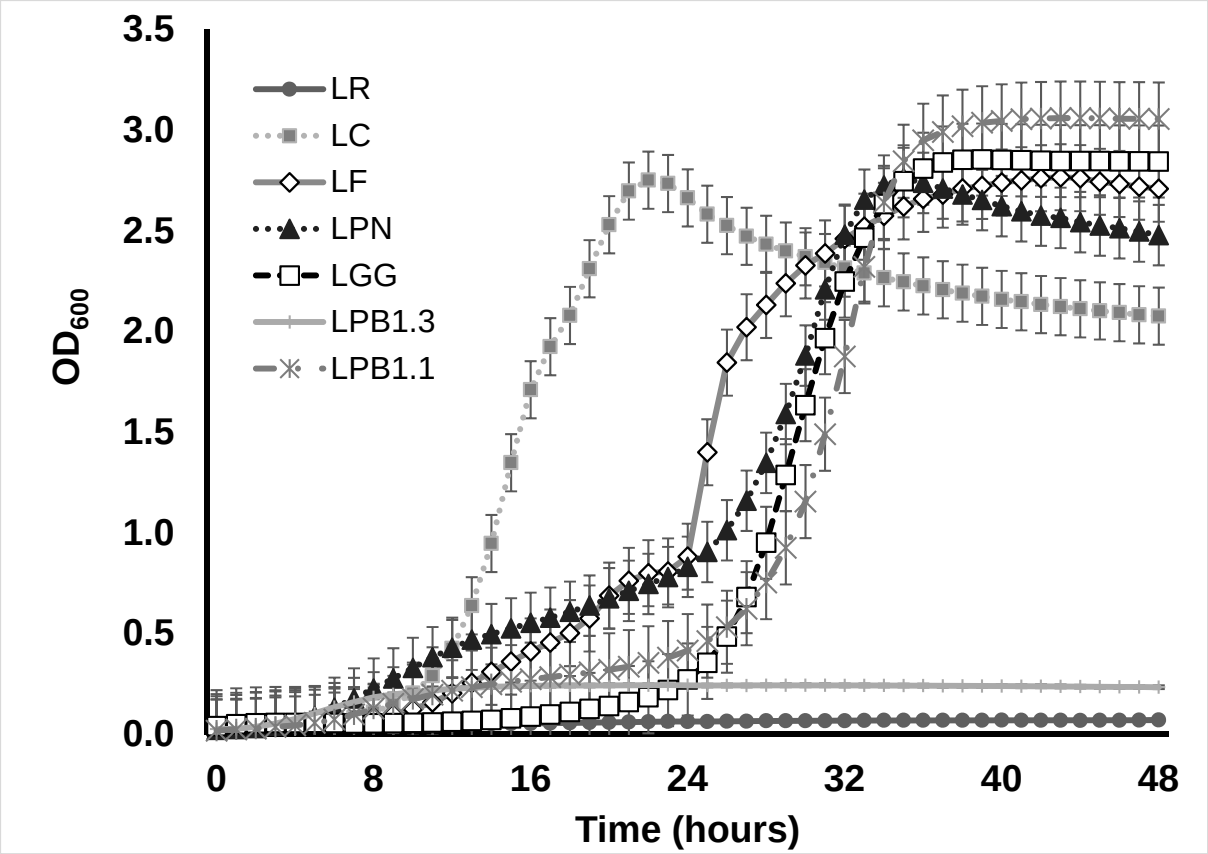

Figure 3. Growth of Lactobacillus spp. on beta-glucan oligosaccharides (BGOs). The growth of seven *Lactobacillus spp.* on the β-glucan oligosaccharides was monitored spectrophotometrically by measurement of the optical density of cells at 600 nm over a 48-hour time span. Each culture was tested in triplicate for all organisms and averaged. Data shown is representative of multiple growth experiments with BGOs. Error expressed as a standard error of triplicate measurements.